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Elevated temperature relieves phosphorus limitation of marine unicellular diazotrophic cyanobacteria

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Abstract

The warming ocean is expected to be more phosphorus (P) limited due to increasing stratification. P is a major limiting nutrient of marine diazotrophs, while the interactive effect of temperature elevation and P limitation on marine unicellular diazotrophs is unknown. Here, we examined the physiology of a major unicellular diazotroph, *Crocosphaera watsonii*, grown under P-limited and P-replete conditions at 25°C, 28°C, and 31°C. Growth, N_2, and CO_2 fixation rates of *C. watsonii* increased with temperature under P limitation, and growth rates were similar between P-limited and P-replete treatments at 31°C. At high temperature, the P use efficiencies for N_2 and CO_2 fixation under P limitation were more than twice higher than under P-replete conditions. Expression of genes involved in P acquisition, intracellular recycling, and substitution in *C. watsonii* was upregulated at higher temperature under P limitation. These results suggest that P limitation in *C. watsonii* was relieved with elevated temperature through various temperature-dependent economic strategies on P metabolism. Through meta-analysis of a field data set using general additive model, we found that *C. watsonii* abundance was correlated mainly with temperature and phosphate, and predicted to increase significantly with further warming.

Nitrogen (N)-fixing (diazotrophic) cyanobacteria play a crucial role in marine biogeochemical cycles, particularly in oligotrophic waters, where diazotrophic cyanobacteria are the major new N source of primary productivity and they mediate carbon (C) and N export of the euphotic zone (Karl et al. 1997; Mulholland 2007). *Trichodesmium* is the most well-studied diazotrophic cyanobacterium, which has long been regarded as a dominant N_2 fixer in the ocean (Capone et al. 1997; Bergman et al. 2013; Zehr and Capone 2020). However, diverse unicellular cyanobacteria were later discovered abundant in tropical and subtropical oceans and conducting equal or more N_2 fixation compared with *Trichodesmium* (Moisander et al. 2010; Martínez-Pérez et al. 2016). Marine unicellular diazotrophic cyanobacteria consist of three phylogenetically distinct groups, including UCYN-A, UCYN-B, and UCYN-C (Zehr 2011). So far, UCYN-B (*Crocosphaera watsonii*) and UCYN-C (*Cyanothece*) have been cultivated (Webb et al. 2009; Taniuchi et al. 2012), but UCYN-B is more abundant than UCYN-C in the open ocean (Shiozaki et al. 2017; Chen et al. 2019).

C. watsonii has been frequently used as a major model organism for understanding the physiology of marine unicellular diazotrophic cyanobacteria (Fu et al. 2014; Masuda et al. 2018; Yamaguchi et al. 2020). To date, culture-based experiments have shown that a number of environmental factors appear to affect the physiology of *C. watsonii*, such as

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temperature and nutrient availability, especially phosphorus (P) and iron (Fe) (Fu et al. 2014; Zhu et al. 2020; Yang et al. 2021). The physiological rates of *C. watsonii* showed unimodal thermal response curves, with the optimal temperatures for growth, CO_2 and N_2 fixation ranging 28–30°C (Fu et al. 2014). Growth and N_2 fixation of *C. watsonii* can be limited by either P (Zhu et al. 2020), or Fe (Yang et al. 2021), as well as the combination of the two (Garcia et al. 2015).

Global warming is projected to lead to about a 3°C increase in the mean sea surface temperature by the end of this century due to the high anthropogenic CO_2 emission (Collins et al. 2013). In particular, ocean warming for the surface waters will be the strongest in the tropical and subtropical regions (Stocker et al. 2013), where the major domains of the marine diazotrophic cyanobacteria are found (Cheung et al. 2020; Zehr and Capone 2020). The increase in sea surface temperature will intensify the stratification of surface ocean and subsequently reduce the replenishment of nutrients (e.g., nitrate and phosphate) from the subsurface water (Doney 2006). The decline of N supply is proposed to limit nondiazotrophic phytoplankton growth but select diazotrophic cyanobacteria that can fix N_2 (Karl et al. 2001). Thus, diazotrophic cyanobacteria may experience more P-impoverished condition in the warming ocean (Hutchins and Fu 2017), especially in oligotrophic waters, where P is mainly delivered to the surface water from deeper water via mixing and advective processes (Karl 2014). P is crucial to the growth and metabolism of organisms since it is a key element in nucleic acids, phospholipids and other important biomolecules (Karl 2014). Many studies have suggested that P limits N_2 fixation by diazotrophs in marine ecosystems (Webb et al. 2007; Sohm et al. 2008; Turk-Kubo et al. 2012). In contrast, atmospheric Fe input into the surface ocean was proposed to increase under the global climate change (Hutchins and Boyd 2016). Hence, marine diazotrophs are expected to face both temperature elevation and more severe P limitation in the projected warming ocean, whereas how they respond to the interaction of temperature elevation and P limitation remains obscure. Considering the ecological importance of the diazotrophic cyanobacteria in supporting the fertility, productivity, and C sequestration of the ocean (Zehr and Capone 2020), it is important to understand the physiological responses of marine diazotrophic cyanobacteria under the proposed conditions in the future ocean.

So far, studies of interactive effects of temperature and P availability on marine diazotrophic cyanobacteria were only limited to *Trichodesmium*, in which different *Trichodesmium* strains showed inconsistent responses to warming under P-limited conditions (Hutchins et al. 2007; Qu et al. 2019). It should be noted that *C. watsonii* and *Trichodesmium* have different growth thermal limits and optimum growth temperatures (Fu et al. 2014). These two N_2 fixers also differ in P metabolism. In contrast to *Trichodesmium*, *C. watsonii* is unable to utilize phosphonates, which roughly account for 25% of the dissolved organic P in the ocean (Clark et al. 1998; Pereira et al. 2019). In addition, due to its smaller cell size, *C. watsonii* has larger cell surface area to volume ratio for the uptake of P compared to *Trichodesmium* (Finkel et al. 2009). Therefore, investigating the physiological responses of unicellular diazotrophic cyanobacteria to temperature elevation and P limitation is in critical need. In addition, none of the previous relevant studies has studied the underlying molecular mechanisms (Hutchins et al. 2007; Qu et al. 2019). Various strategies for overcoming P limitation have been proposed in cyanobacteria (Pereira et al. 2019), including high-affinity scavenging for extracellular inorganic phosphate, utilizing organic P compounds, and lowering cellular P requirement by element substitution (Dyhrman and Haley 2006; Van Mooy et al. 2006; Pereira et al. 2019). It has also been reported that *C. watsonii* has versatile genetic potential of P acquisitions (Pereira et al. 2019). However, it is uncertain whether/how temperature would affect the processes of P metabolism and hence mediate the physiological responses of *C. watsonii* to P limitation in a warming ocean.

In order to better predict the fate of unicellular diazotrophic cyanobacteria as well as N_2 fixation in the future climate scenario, the response of *C. watsonii* to the interactive effects of temperature elevation and P limitation was resolved with both laboratorial experiments and a meta-analysis of a field observational data set in the current study. We examined the physiological responses including growth and N_2 and CO_2 fixation rates, as well as the molecular responses (i.e., the expression of P metabolism related genes) of *C. watsonii* under P-limited (200 nmol L^{-1}) and P-replete (45 μmol L^{-1}) conditions at three temperatures (25°C, 28°C, and 31°C). Besides, a meta-analysis on a global data set of *C. watsonii* abundance was also conducted to provide additional evidence of the effects of temperature and P availability on *C. watsonii* in the real ocean.

Materials

Cell culturing and experimental design

C. watsonii WH8501 cultures were maintained in N-free Scripps Oceanographic medium (Tuit et al. 2004), prepared with 0.2 μm filtered artificial seawater and 36 μmol L^{-1} Na_2HPO_4. Cultures were grown in polycarbonate bottles at the light intensity of 80 μmol photons m^{-2} s^{-1} with a 12 : 12-h light : dark cycle. Cells were acclimated at three temperatures (25°C, 28°C, and 31°C) and diluted with fresh medium every 2 weeks for at least 6 months. To acclimate the cultures to P-limited conditions, we transferred half volume of cultures to the equal volume of fresh P-limited (200 nmol L^{-1} phosphate) medium every 3 d. After repeating that for a number of times and the culture reached the targeted phosphate concentration, each bottle of culture was diluted every 3–4 d to the initial cell density of around 5×10^4 cells mL^{-1} with fresh P-limited medium. The cell abundance was checked using a Becton–

Dickinson FACSCalibur flow cytometer. Acclimation was confirmed by steady-state growth for at least three generations (no significant difference in growth rates). The acclimation of P-replete (45 μmol L^{-1} phosphate) cultures was conducted in the same as P-limited cultures.

After acclimation, the cultures were grown in triplicates of 500-mL polycarbonate bottles under six treatments that consisted of factorial combinations of two phosphate concentrations (P-limited, 200 nmol L^{-1} phosphate; and P-replete, 45 μmol L^{-1} phosphate) and three temperatures (25°C, 28°C, and 31°C). Growth rates were determined during a 3-d growth cycle, while N_2 and CO_2 fixation rates, particulate C, N and P, and cell size were measured at the end of a 3-d growth cycle.

Growth rate measurement

Growth rates of *C. watsonii* WH8501 were calculated by determining the changes in cell abundance during a 3-d growth cycle. Growth rate (μ, d^{-1}) was calculated as: $\mu = \ln(C_2/C_1)/(t_2 - t_1)$, where C_2 and C_1 were the cell abundances (cells mL^{-1}) at t_2 and t_1, respectively. The samples (1.8 mL) for determining the cell abundance were collected from each bottle, fixed with 50 μL 20% paraformaldehyde solution (Guo et al. 2014), and preserved at −80°C until analysis with a Becton–Dickinson FACSCalibur flow cytometer.

N_2 and CO_2 fixation rates measurement

N_2 and CO_2 fixation rates were determined using the $^{15}N_2$ gas tracer and ^{13}C-tracer addition method, respectively (Hama et al. 1983; Montoya et al. 1996; Mulholland and Bernhardt 2005). Briefly, 3 h before the onset of the dark period, each 60-mL serum bottle was filled with culture samples from a treatment replicate but leaving some headspace and 64 μL of 0.2 mol L^{-1} $NaH^{13}CO_3$ (Sigma-Aldrich) was added to each bottle. Serum bottles were then topped up with cultures. After closing the bottles with Telfon-coated butyl rubber septum caps, 0.5 mL of $^{15}N_2$ gas (Cambridge Isotope Laboratories) was injected into each serum bottle with a gas-tight syringe. All the samples were incubated for 24 h at designated temperatures. After incubations, the samples were filtered onto precombusted (550°C, 5 h) GF/A filters (Whatman) and stored at − 80°C before analysis using a continuous flow isotope ratio mass spectrometer (CF-IRMS; Isoprime, GV Instruments). N_2 and CO_2 fixation rates were calculated as described in Montoya et al. (1996) and then normalized to total biomass of each sample given that the cell size of *C. watsonii* changed with temperature and phosphate concentration.

Elemental analysis

Cellular particulate organic C and N were determined together with their isotopic compositions using a continuous flow isotope ratio mass spectrometer coupled with an elemental analyzer (Eurovector 3000 Series). For the measurement of particulate organic P, 50/100 mL subsamples from each treatment replicate were filtered onto precombusted (550°C, 5 h) GF/A filters (Whatman) and stored at − 80°C before analysis using wet oxidation method (Pujo-Pay and Raimbault 1994).

Phosphorus use efficiency

The phosphorus use efficiencies (PUEs) for N_2 and CO_2 fixation were defined as the quantity of N_2 or CO_2 fixed per unit time per unit cellular P, respectively (Qu et al. 2019). In the calculation of N-specific and C-specific PUEs, N_2 and CO_2 fixation rates were normalized to cellular particulate organic P, respectively.

Cell size measurement

The cell size of *C. watsonii* WH8501 at each treatment was measured by the microscopic observation. To avoid the effects of diel variation in cell size of *C. watsonii* (Mohr et al. 2010), samples from different treatments were collected simultaneously. Samples (1 mL) collected from each treatment were fixed with glutaraldehyde (0.5% final concentration) and then filtered onto 0.8-μm pore size mixed cellulose esters membrane (Sangon Biotech) dyed with Sudan Black B. The filters were mounted on glass slides and stored at − 20°C. Cell diameters were determined using an Olympus BX51 microscope (Olympus) equipped with a camera at ×1000 magnification. Images were analyzed with SPOT Advance software (Diagnostic Instruments). The cell diameters of more than 15 cells were observed and measured.

Quantifying expression levels of the genes involved in P metabolism

Given that the expression of the genes involved in P metabolism in *C. watsonii* exhibited a diel pattern (Shi et al. 2010), RNA samples were collected from each treatment replicate in the middle of light and dark period, respectively. The samples (50 mL) were filtered onto 1-μm pore size polycarbonate filter membranes under low vacuum pressure. Each membrane sample was then placed in a sterilized centrifuge tube and immediately stored at − 80°C after adding 1 mL TRIzol Reagent (Ambion, Life Technologies). RNA was extracted using the TRIzol Reagent and PureLink RNA Mini Kit (Ambion, Life Technologies) and subsequently reverse-transcribed into cDNA using HiScript® III RT SuperMix for quantitative polymerase chain reaction (qPCR) with gDNA wiper (Vazyme) according to the manufacturers' protocols.

Relative transcript abundances of the genes involved in P metabolism were quantified using qPCR assays with the primers designed in a previous study (Pereira et al. 2016). We examined the transcription of 10 P metabolism related genes that involved in gene regulation (*phoB*, *phoU*, and *phoH*), P scavenging (*pstS*, *sphX*, and *phoA*), substitution (*sqdB*), and recycling (*ppX*, *ppK*, and *ppA*) in response to P limitation at different temperatures. Each qPCR reaction was run with SYBR Green SuperMix (Roche Diagnostics GmbH), containing 5 μL

SYBR Green SuperMix, 1 μL forward and reverse primers (10 pmol/μL), 3.5 μL ultrapure distilled water (Invitrogen, Thermo Fisher Scientific Corp.), and 0.5 μL cDNA. The reactions were run with triplicates in 384-well plates on a Light Cycler 480 (Roche Diagnostics GmbH) with the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. No-template controls were run in triplicates during each qPCR. The $2^{-\Delta\Delta C_t}$ method was used to normalize the expression level of each gene (Livak and Schmittgen 2001). The threshold cycle (Ct) value of each gene was normalized to a housekeeping gene (*rnpB*, encoding the RNA component of RNase P), whose transcript abundance was found not to vary under different nutrient and temperature conditions in marine cyanobacteria (Gómez-Baena et al. 2009; Chappell and Webb 2010). A previous study also showed that the transcription of *rnpA* gene (encoding the protein component of RNase P) in *C. watsonii* WH8501 does not show a diel variation (Shi et al. 2010).

Analyzing the *nifH* gene abundance of *C. watsonii* in field using general additive model

We used general additive model (GAM) to analyze the relationship between the *nifH* gene abundance of *C. watsonii* and environmental factors in the global ocean using a data set compiled by Tang and Cassar (2019). This data set contains *nifH* gene abundance of *C. watsonii* estimated by qPCR using the DNA samples collected from the Pacific Ocean, Atlantic Ocean, Indian Ocean, and their marginal seas, together with the corresponding environmental factors (i.e., temperature, phosphate, nitrate, and nitrate to phosphate ratio) (Tang and Cassar 2019). The environmental factors that showed significant relationships ($p < 0.05$) with the *nifH* gene abundance of *C. watsonii* were selected as the predictors for further constructing a GAM to predict the *nifH* gene abundance of *C. watsonii*. The data set of *C. watsonii nifH* gene abundance was partitioned randomly into training (90%) and testing (10%) data sets using package caTools in R (Tuszynski 2012). The training data set was used to construct the model. The environmental factors in the testing data set were input into the model to predict the corresponding *C. watsonii nifH* gene abundance. The observed and predicted values of the *C. watsonii nifH* gene abundance of the testing data set were compared to evaluate the predictive accuracy of the model. To estimate the changes of *C. watsonii nifH* gene abundance under warming and P limitation, we predicted *C. watsonii nifH* gene abundance with altered environmental predictors using this model, in which the temperature and phosphate concentration were increased by 4°C and reduced by five times, respectively. The GAM analysis was conducted using the package "mgcv" in R (Wood and Wood 2015).

Statistical analysis

Two-way ANOVA was used to test the significance of the differences in the interactive effects of temperature and phosphate concentration on *C. watsonii*, and one-way ANOVA was applied to test the significance of the differences for both P levels among different temperatures. The Tukey multiple comparison test was used to determine the significance of the differences between individual treatments. All statistical analyses were performed with a SPSS 26.0 software. The significance level of 0.05 was set.

Results

Growth rates and cell size of *C. watsonii*

Growth rates of *C. watsonii* WH8501 under P-limited conditions were significantly lower than under P-replete conditions regardless of the temperature (two-way ANOVA, $p < 0.001$), indicating that the low phosphate concentration inhibited the growth of *C. watsonii* (Fig. 1A). Under P-replete conditions, growth rates of *C. watsonii* at 31°C were significantly lower

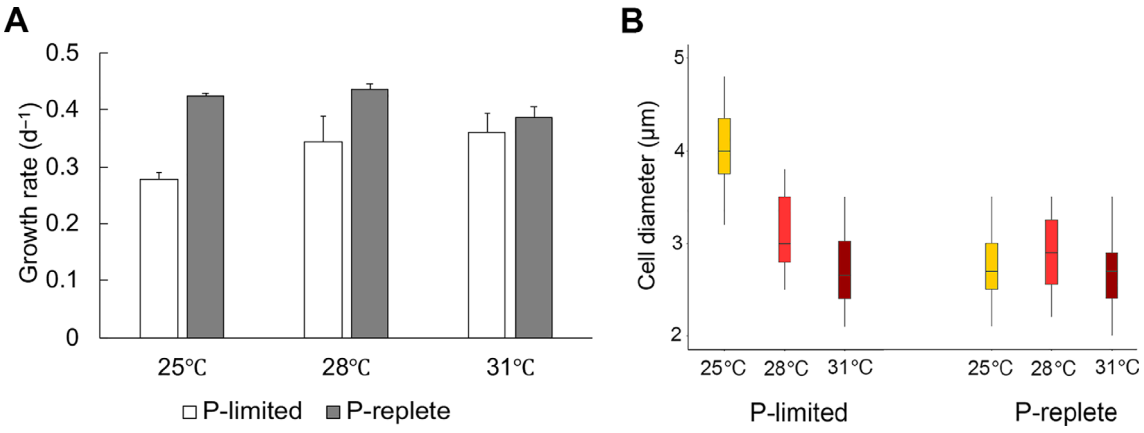

Fig. 1. (**A**) Growth rates (d^{-1}) and (**B**) cell size (μm) of *Crocosphaera watsonii* WH8501 under P-limited and P-replete conditions (200 nmol L^{-1} and 45 μmol L^{-1}) at three temperatures (25°C, 28°C, and 31°C). Given morphological data (e.g., cell size) were generally more variable than the physiological data (e.g., growth rates), cell diameters of *C. watsonii* were shown as a box plot. Values denote mean ± standard deviation of biological replicates under each treatment ($n = 3$ for growth rates, $n \geq 15$ for cell size).

than at 25°C and 28°C ($p < 0.05$), with the highest growth rates at 28°C (Fig. 1A). In contrast, the growth rates of *C. watsonii* increased with rising temperature (i.e., from 25°C to 31°C) under P-limited conditions (Fig. 1A). Meanwhile, the differences among the growth rates under P-limited and P-replete treatments decreased with increasing temperature. Specifically, P limitation lowered the growth rates of *C. watsonii* by 35% at 25°C, 21% at 28°C and 6% at 31°C, which suggested that elevated temperature lessened the effects of P limitation and almost relieved P limitation on the growth of *C. watsonii* at 31°C (Fig. 1A).

Both the temperature (two-way ANOVA, $p < 0.01$) and P availability (two-way ANOVA, $p < 0.01$) had a significant effect on cell diameters (μm) of *C. watsonii*. Increased temperature resulted in a decrease of cell size in the P-limited cultures, with the cell diameters decreasing from 25°C to 28°C by 23% and to 31°C by 33% (Fig. 1B). However, the cell diameters in the P-replete cultures did not vary significantly among different temperatures ($p > 0.05$, Fig. 1B). At 25°C, the cell diameters in the P-limited cultures were much larger than those in the P-replete cultures ($p < 0.05$, Fig. 1B), while there was no significant difference between these two P levels at 28°C and 31°C ($p > 0.05$, Fig. 1B).

N_2 and CO_2 fixation rates

Both temperature (two-way ANOVA, $p < 0.01$) and P availability (two-way ANOVA, $p < 0.05$) significantly affected N_2 fixation rates of *C. watsonii*. Under P-limited and P-replete conditions, elevated temperature resulted in an increase in N_2 fixation rates, but no significant difference was observed among different temperatures under P-replete conditions ($p > 0.05$, Fig. 2A). In contrast, under P-limited conditions, N_2 fixation rates did not change significantly from 25°C to 28°C ($p > 0.05$) and increased by 246% from 25°C to 31°C ($p < 0.05$, Fig. 2A). Thus, as with growth rates, elevated temperature greatly enhanced the N_2 fixation capabilities of *C. watsonii* under P-limited conditions.

CO_2 fixation rates were also affected by temperature (two-way ANOVA, $p < 0.01$) and P availability (two-way ANOVA, $p < 0.001$). Under P-limited conditions, CO_2 fixation rates increased greatly by 495% from 25°C to 31°C ($p < 0.05$), though the difference between 28°C and 31°C was not significant ($p > 0.05$, Fig. 2B). Under P-replete conditions, CO_2 fixation rates reached a maximum at 28°C, significantly higher than at 25°C and 31°C ($p < 0.05$, Fig. 2B). Hence, elevated temperature also had a stronger positive effect on the CO_2 fixation capabilities of *C. watsonii* under P-limited conditions

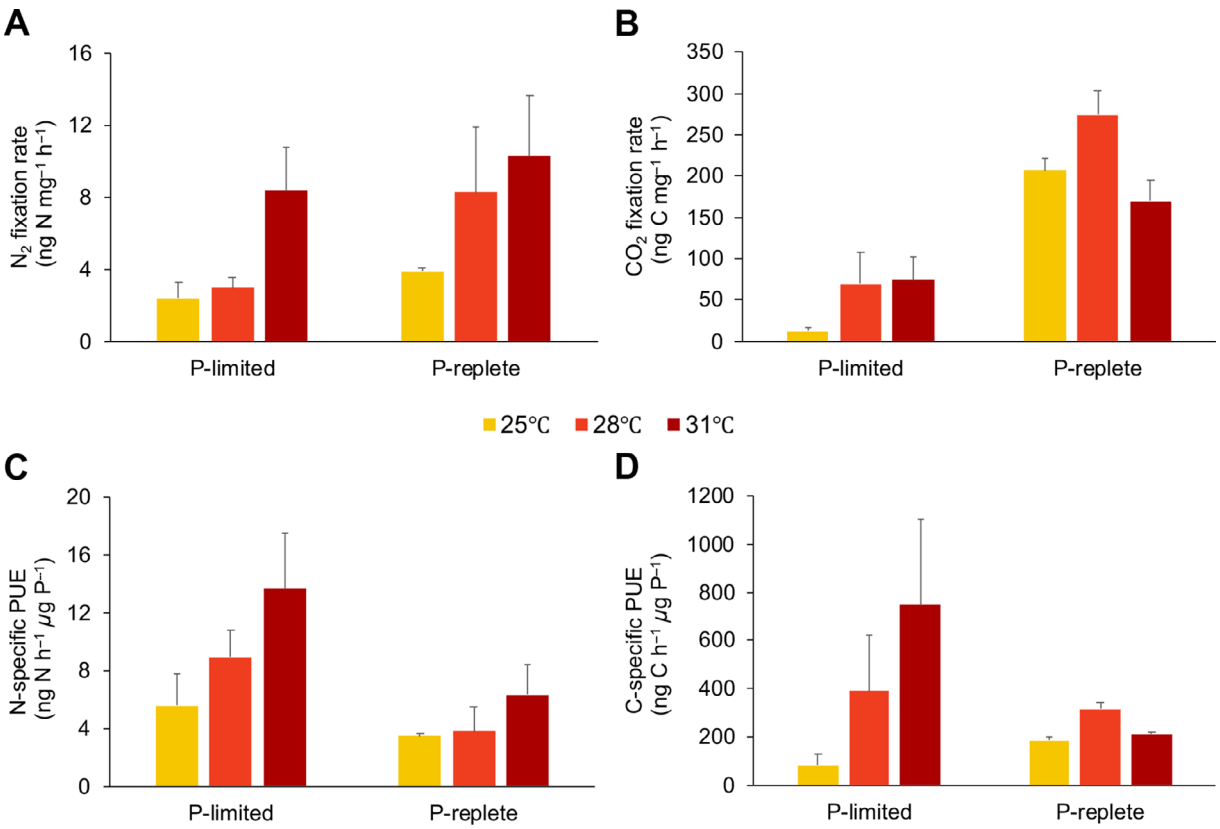

Fig. 2. (**A**) N_2 fixation rates (ng N mg^{-1} h^{-1}), (**B**) CO_2 fixation rates (ng C mg^{-1} h^{-1}), (**C**) N-specific PUE (ng N h^{-1} μg P^{-1}), (**D**) C-specific PUE (ng C h^{-1} μg P^{-1}) of *Crocosphaera watsonii* WH8501 under P-limited and P-replete conditions (200 nmol L^{-1} and 45 μmol L^{-1}) at three temperatures (25°C, 28°C, and 31°C). Values denote mean ± standard deviation of biological replicates under each treatment ($n = 3$).

than under P-replete conditions. The differences of CO_2 fixation rates between P-limited and P-replete cultures were larger than those of N_2 fixation rates (Fig. 2A,B).

Phosphorus use efficiency

The PUE for N_2 fixation (ng N h^{-1} μg^{-1} P) under P limitation was higher than under P-replete conditions regardless of the temperature (two-way ANOVA, $p < 0.05$, Fig. 2C). Under P limitation, the PUE for N_2 fixation increased slightly from 25°C to 28°C ($p > 0.05$) and increased significantly by 144% from 25°C to 31°C ($p < 0.05$, Fig. 2C). Under P-replete conditions, the PUE for N_2 fixation increased slightly with rising temperature, although no significant difference was observed among different temperatures ($p > 0.05$, Fig. 2C). The PUE for CO_2 fixation (ng C h^{-1} μg^{-1} P) increased by eightfold from 25°C to 31°C in the P-limited cultures ($p < 0.05$, Fig. 2D). However, under P-replete conditions, the PUE for CO_2 fixation was the highest at 28°C, which was significantly higher than that at 25°C and 31°C ($p < 0.05$, Fig. 2D). For both N_2 and CO_2 fixation, the PUEs showed thermal response patterns similar to the corresponding rates (Fig. 2).

Expression levels of P metabolism genes

Comparing with the P-replete cultures, the genes related to P scavenging (*pstS*) and the associated regulator (*phoU*) were significantly upregulated in the P-limited cultures at all tested temperatures ($p < 0.05$, Fig. 3A). Specifically, the expression of *pstS* gene increased 3- to 13-fold under P-limited conditions, while the expression levels of *phoU* gene in the P-limited cultures were about 4-fold higher than in the P-replete cultures (Fig. 3A). The expression of another P scavenging gene (*sphX*) increased significantly in the P-limited cultures compared to the P-replete cultures at high temperature (i.e., threefold at 28°C and eightfold at 31°C) ($p < 0.05$, Fig. 3A). The genes involved in degradation of polyphosphate (polyP) (*ppX*) and regulation of phosphate transport (*phoB*) were only upregulated in the P-limited cultures at 31°C ($p < 0.05$, Fig. 3A).

To explore at the molecular level how *C. watsonii* responds to ocean warming under P limitation, we compared the expression levels of the genes involved in P metabolism with rising temperature under P-limited and P-replete conditions (Figs. 3B,C). With increasing temperature, P-limited *C. watsonii* significantly upregulated several genes involved in P scavenging (*pstS* and *sphX*) and substitution (*sqdB*), gene regulation (*phoB* and *phoH*), and intracellular P recycling (*ppA* and *ppK*) ($p < 0.05$, Fig. 3B). The expression levels of these genes were twofold to fourfold higher at 28°C and 31°C than at 25°C under P limitation (Fig. 3B). The expression of the genes involved in regulating the phosphate transport (*phoU*) and hydrolyzing dissolved organic P (*phoA*) did not change significantly with increasing temperature under P limitation ($p > 0.05$, Fig. 3B). Under P-replete conditions, *ppA* gene was significantly upregulated with increasing temperature

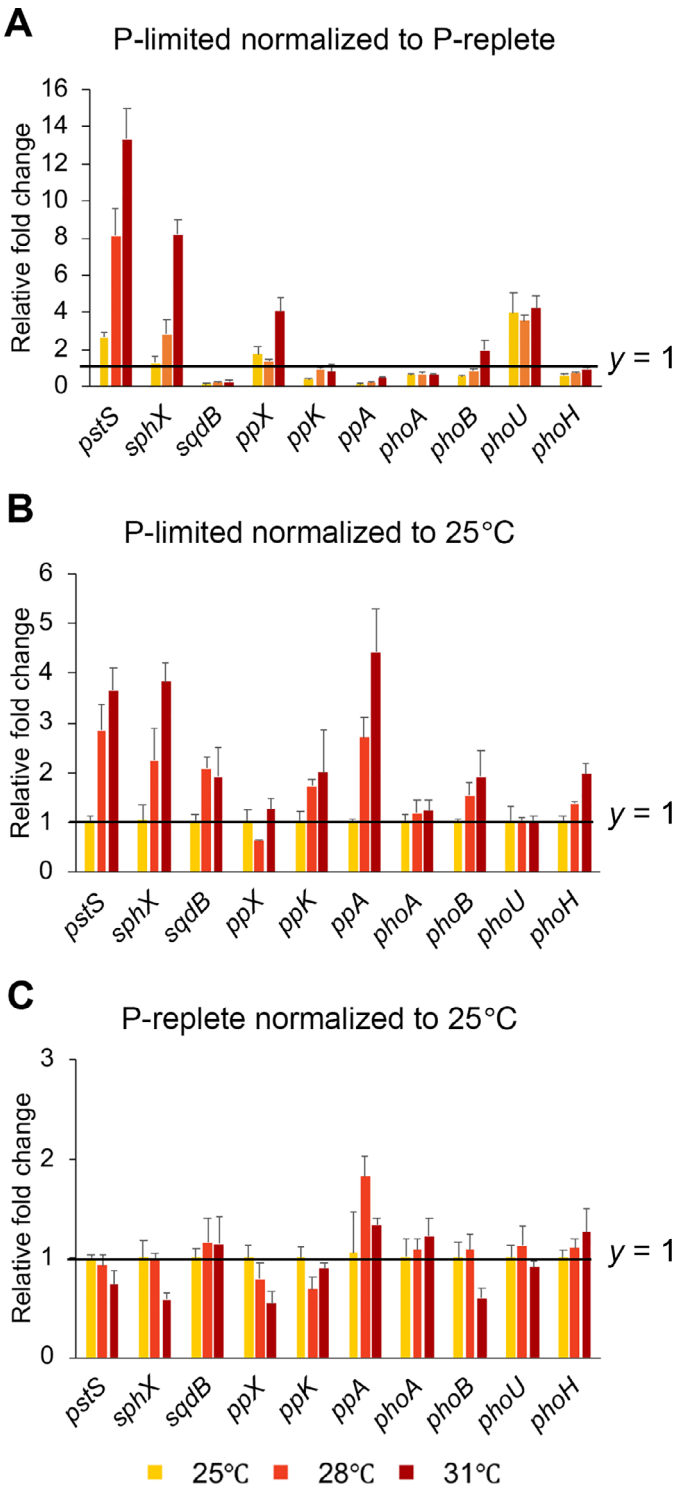

Fig. 3. (**A**) Relative fold changes (with *rnpB* gene as the reference gene, P-limited normalized to P-replete) of expression of key P metabolism genes in *Crocosphaera watsonii* WH8501 at three temperatures (25°C, 28°C, and 31°C). (**B**) Relative fold changes (with *rnpB* gene as the reference gene, 28°C, 31°C normalized to 25°C) of expression of key P metabolism genes in *C. watsonii* WH8501 under P-limited conditions (200 nmol L^{-1}). (**C**) The same as (**B**), but for P-replete conditions (45 μmol L^{-1}). Values denote mean ± standard deviation of biological replicates under each treatment ($n = 3$).

($p < 0.05$, Fig. 3C), while the genes involved in P scavenging (*pstS* and *sphX*), polyP regeneration (*ppX* and *ppK*) and transcriptional regulation (*phoB*) were downregulated with increasing temperature ($p < 0.05$, Fig. 3C).

Environmental determinants and prediction of the *nifH* gene abundance of *C. watsonii* in field

Among all the tested physicochemical factors, temperature and phosphate showed significant relationships with the *nifH* gene abundance of *C. watsonii* as revealed by the GAM, in which these two factors explained 73.2% variation of the *nifH* gene abundance of *C. watsonii*. Temperature showed a positive and strong relationship with the *nifH* gene abundance of *C. watsonii* (p value $< 2 \times 10^{-16}$), while phosphate showed a slightly negative correlation with the *nifH* gene abundance of *C. watsonii* (p value $= 0.0086$) (Figs. 4A,B). These two factors were further used for constructing a GAM (*C. watsonii nifH* gene abundance $\sim$ s [temperature] + s [phosphate]) that was used to predict the *nifH* gene abundance of *C. watsonii* under the ambient, high temperature (temperature + 4°C; phosphate concentration unchanged) and high temperature and low phosphate (temperature + 4°C; phosphate concentration reduced by five times) conditions. The predicted *nifH* gene abundance of *C. watsonii* with the ambient temperature and phosphate concentration was similar to the observed *nifH* gene abundance in both training ($r^2 = 0.72$) and testing ($r^2 = 0.66$) data sets. When the temperature was increased by 4°C, the average *nifH* gene abundance of *C. watsonii* was predicted to increase from 10^7 to 10^8 *nifH* gene copies m^{-3} (Fig. 4C). Under the high temperature conditions (+ 4°C), P limitation was predicted to slightly increase the *nifH* gene abundance of *C. watsonii* (Fig. 4C).

Discussion

Interactive effects of warming and P limitation on the physiological rates of *C. watsonii*

As revealed by the incubation experiments, P limitation had a negative impact on growth, N_2 and CO_2 fixation rates of *C. watsonii* in general (Figs. 1, 2), which agreed with previous studies (Garcia et al. 2013*b*; Zhu et al. 2020). However, we found that these physiological rates of *C. watsonii* increased with temperature under P limitation. As such, the growth rates of *C. watsonii* under P-limited and P-replete conditions became similar at 31°C (Fig. 1A). Moreover, the PUEs for N_2 and CO_2 fixation in *C. watsonii* increased with temperature under P limitation (Fig. 2C,D). These results suggested that the impact of P limitation on *C. watsonii* is temperature dependent and high temperature (31°C) can relieve the P limitation of *C. watsonii*. Similarly, warming was also found to promote growth, CO_2

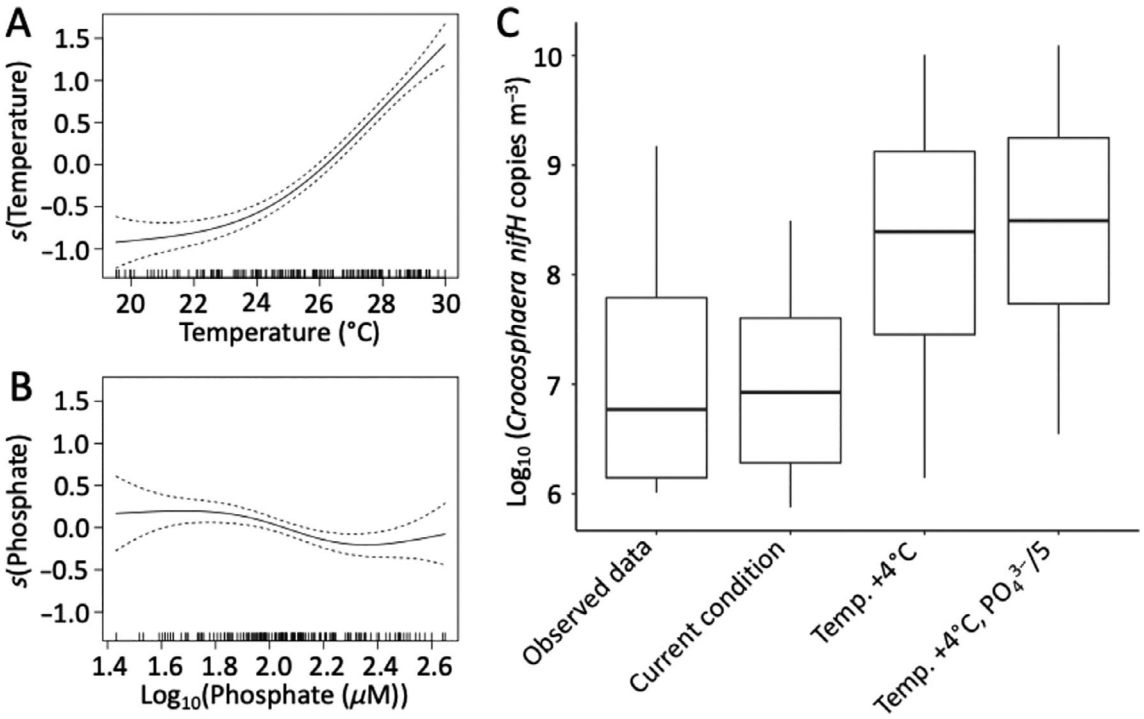

Fig. 4. Partial effects of (**A**) temperature and (**B**) phosphate concentration on the abundance of *Crocosphaera watsonii* (*nifH* gene abundances) as revealed by a GAM (*C. watsonii nifH* gene abundance $\sim$ s (temperature) + s (phosphate); $r^2 = 0.72$; deviance explained = 73.2%). (**C**) Predicted abundances of *C. watsonii* at current (ambient temperature and phosphate concentration), high temperature (temperature + 4°C; phosphate concentration unchanged), and high temperature and low phosphate conditions (temperature + 4°C; phosphate concentration reduced by five times). The prediction was conducted using the GAM.

and N_2 fixation rates of *Trichodesmium erythraeum* GBRTRLI101 under P limitation (Qu et al. 2019), despite many physiological differences between *Trichodesmium* and *C. watsonii* (e.g., optimal growth temperature, thermal range, P metabolism, and cell size). Besides, although growth rates were similar between P-limited and P-replete cultures at high temperature (Fig. 1A), CO_2 fixation rates were still low in P-limited cultures relative to the other physiological rates at high temperature (Fig. 2B). These results suggested that growth and CO_2 fixation may not be closely coupled in *C. watsonii* in response to P limitation. In other words, *C. watsonii* does not require as much C as it fixes under P-replete conditions for survival. It has been found that *C. watsonii* formed carbohydrate granules for storing excessively fixed C under nutrient sufficient conditions (Dron et al. 2012). In contrast, the inhibitory effect of P limitation on N_2 fixation was relatively milder compared to CO_2 fixation (Fig. 2). It has also been reported that N_2 and CO_2 fixation were not tightly coupled or co-regulated in diazotrophic cyanobacteria (Gallon et al. 2002).

We compared our findings with another study that investigated the thermal effects on Fe limitation in *C. watsonii* (Yang et al. 2021). Higher temperatures enhanced growth, CO_2 and N_2 fixation rates of *C. watsonii* under both Fe and P limitation. In addition, warming increased PUEs and Fe use efficiencies for N_2 and CO_2 fixation under P and Fe limitation, respectively, with the peaks corresponded with optimal growth temperatures. Collectively, these findings suggested that warming may increase the efficiency of P and Fe utilization of *C. watsonii*, relieve the limitation of both nutrients, and thus allow *C. watsonii* to overcome the oligotrophic conditions in the future ocean. This also agreed with the result of meta-analysis that the *nifH* gene abundance of *C. watsonii* in field was positively correlated with temperature (Fig. 4). Nevertheless, some differences in the thermal responses of *C. watsonii* were also observed between these two studies. For example, under Fe limitation, the growth, CO_2 and N_2 fixation rates of *C. watsonii* increased from 22°C to 27°C and then decreased as temperature further increased from 27°C to 32°C (Yang et al. 2021). In contrast, these physiological rates increased continuously from 25°C to 31°C under P limitation (Figs. 1A, 2). In addition, PUEs for N_2 and CO_2 fixation were the highest at 31°C under P limitation, while Fe use efficiencies were the highest at 27°C under Fe limitation. These differences implied that the underlying mechanisms that relieve the P and Fe limitation of *C. watsonii* at elevated temperature might be different. Therefore, omics approaches (e.g., metatranscriptomics and proteomics) are needed to examine and compare the thermal responses of *C. watsonii* under P and Fe limitation. In addition, the differences between these two studies could also be also due to that different strains of *C. watsonii* were used. *C. watsonii* consists of two distinct phenotypic groups differentiated with cell size (Webb et al. 2009). It has been reported that the large cell group (e.g., WH0005, used in Yang et al. 2021) is equipped with genetic capabilities that are absent from the small cell group (e.g., WH8501, used in this study), such as P metabolism and Fe stress response genes (Bench et al. 2016). Hence, more strains of *C. watsonii* should be tested in the future studies to better understand the physiological responses of this important N_2 fixer to the warmer and more oligotrophic environment of the future ocean.

In addition to *C. watsonii*, the P and Fe limitation of *Trichodesmium* were also found relieved at high temperature, which were also attributed to thermally driven increases in PUEs and Fe use efficiencies (Jiang et al. 2018; Qu et al. 2019). All these results collectively explained why diazotrophic cyanobacteria are thriving in warm and oligotrophic waters (Sohm et al. 2011) and implied that diazotrophic cyanobacteria could be selected and flourishing in a future warmer ocean. It is projected that *Trichodesmium* and *C. watsonii* could increase their N_2 fixation rates by $\sim$ 22% and $\sim$ 91%, respectively, from 2010 to 2100 under the IPCC RCP 8.5 warming scenario, thereby increasing the relative importance of *C. watsonii* as new N source in the future warming of Fe-limited ocean (Jiang et al. 2018; Yang et al. 2021). Additional research efforts are needed to predict the responses of N_2 fixation rates of different diazotrophic cyanobacteria to P-limited and even Fe and P co-limited conditions in the warming ocean.

Molecular mechanisms for relieving P limitation in response to warming

As revealed by qPCR analysis, the expression of the genes involved in P scavenging and metabolism was influenced by both P availability and temperature (Fig. 3), suggesting the potential underlying mechanisms that led to the alleviation of P limitation of *C. watsonii* under elevated temperature (Fig. 5). Under P limitation, the expression of high-affinity phosphate transporters (*pstS* and *sphX*) and the associated transcriptional regulator (*phoB*) was upregulated as temperature increased (Figs. 3, 5). The *phoB* regulates transcription of the genes encoding high-affinity phosphate transporters in response to P availability (Wanner 1993; Reistetter et al. 2013). Hence, our result implied that elevated temperature increases the dissolved inorganic P uptake through high-affinity phosphate transporters (*pstS* and *sphX*) and associated transcriptional regulator (*phoB*) under P limitation (Fig. 5). Besides, the transcription of *sqdB* gene that encodes sulfolipid biosynthesis protein was upregulated with temperature under P-limited conditions (Figs. 3, 5). Sulfolipid was reported to substitute the P-containing phospholipid in cyanobacteria under P-limited conditions (Van Mooy et al. 2009). Although our results showed that P limitation did not upregulate the expression of *sqdB* gene in *C. watsonii* at normal growing temperature, which was consistent with previous studies (Van Mooy et al. 2009; Pereira et al. 2016), rising temperature can boost up the expression of *sqdB* gene under P limitation (Fig. 3). *C. watsonii* could reduce their P requirement by enhancing the

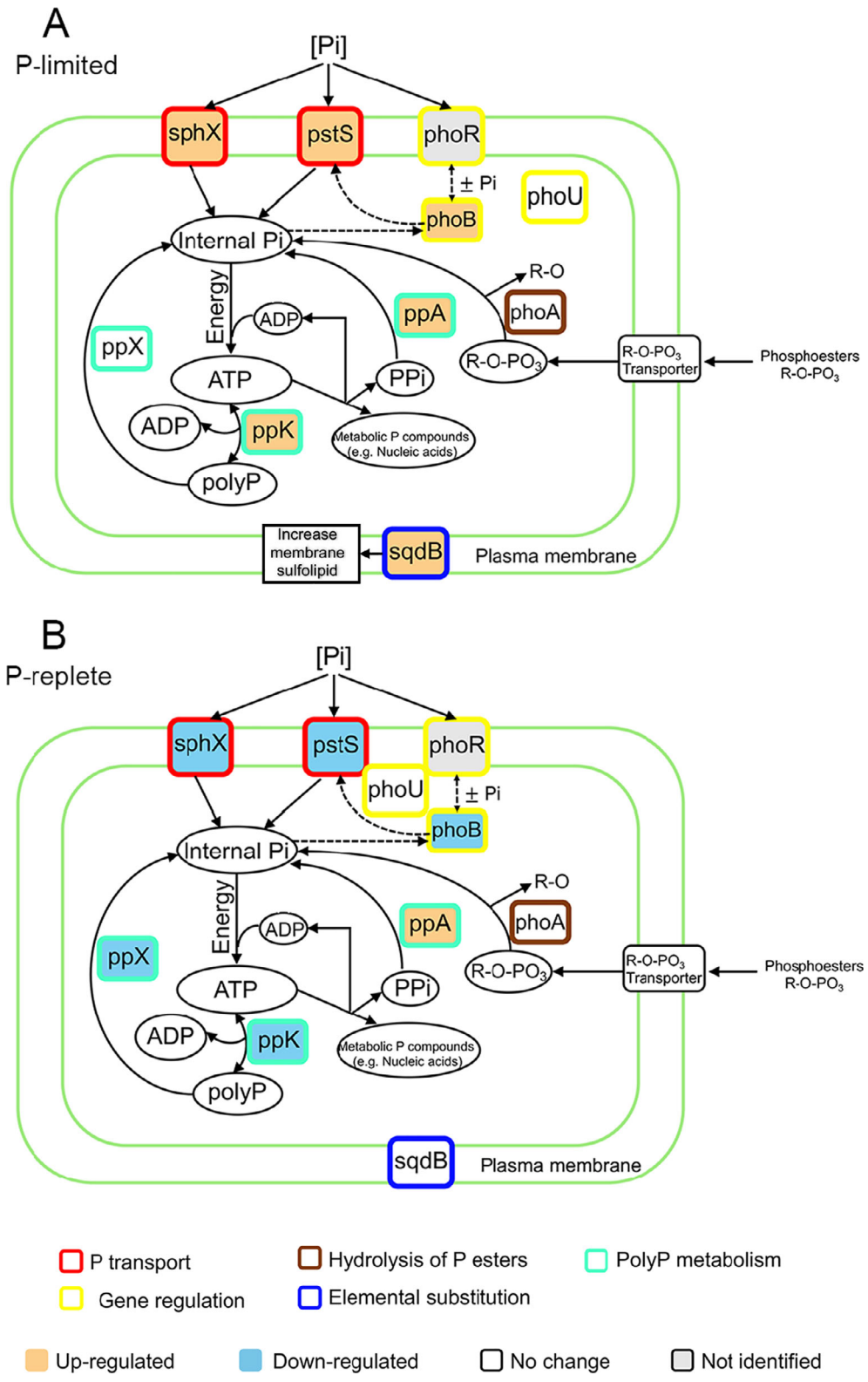

Fig. 5. Schematic illustration showing the regulation of P metabolism genes in *C. watsonii* WH8501 in response to warming under (**A**) P-limited (200 nmol L^{-1}) and (**B**) P-replete conditions (45 μmol L^{-1}). We suggest that enhanced P acquisition, substitution, and intracellular cycling help to relieve P limitation at high temperature. Colors at the periphery of the gene pictograms denote different P metabolisms, and those in the center denote upregulation or downregulation of the P metabolism genes.

substitution of sulfolipid for phospholipid under higher temperatures (Fig. 5). Moreover, the transcription of the genes for intracellular P recycling (*ppK* and *ppA*) increased with rising temperature under P limitation (Figs. 3, 5). In cyanobacteria, the polyP kinase *ppK* can hydrolyze ATP into polyP (serves as cellular phosphate pool) as well as form ATP from polyP (Lin et al. 2016). The *ppA* gene (encoding inorganic pyrophosphatase) is responsible for the pyrophosphate hydrolysis (Gómez-García et al. 2003). The upregulation of *ppK* and *ppA* suggested that the increasing utilization and recycling of intracellular P may be a key strategy for *C. watsonii* to cope with P stress at high temperature (Fig. 5). In contrast, Pscavenging (*pstS*, *sphX*, and *phoB*) and polyP metabolism genes (*ppX* and *ppK*) were downregulated with increasing temperature under P-replete conditions (Figs. 3, 5). It could be because that *C. watsonii* does not need to enhance P scavenging and metabolism under P-replete conditions.

The growth rate hypothesis proposes that organisms with higher growth rates require greater P rich ribosomal RNA to sustain rapid protein synthesis rates (Elser et al. 1996; Sterner and Elser 2002; Gillooly et al. 2005). According to the growth rate hypothesis, the P requirement of fast-growing organisms is expected to be increased. Higher growth rates under high temperature would lead to stronger P limitation of the organisms. However, a later hypothesis (the temperature-dependent physiology hypothesis; Toseland et al. 2013; Yvon-Durocher et al. 2015) proposes that the cellular P requirement and metabolism are also influenced by temperature. In the current study, our results suggested that P acquisition, substitution, and intracellular cycling in *C. watsonii* were upregulated by warming under P-limited conditions (Fig. 5), which explained why PUEs of *C. watsonii* increased and hence P limitation was relieved at high temperature.

Implications for *C. watsonii* in future ocean

Our study found that P limitation on the physiological rates of *C. watsonii* is relieved at elevated temperature. We further proposed, based on the transcription of genes involved in P metabolism, that *C. watsonii* evolved multiple temperature-dependent strategies to overcome P limitation in the ocean (Fig. 5). Analysis of existing field data by the GAM revealed that the *nifH* gene abundance of *C. watsonii* in global ocean was positively correlated with temperature and slightly negatively correlated with phosphate (Figs. 4A,B), indicating that *C. watsonii* is more abundant under warm and P-depleted conditions. We then used the GAM to estimate that the *nifH* gene abundance of *C. watsonii* will increase significantly with further increasing temperature and P limitation (Fig. 4C). Hence, both laboratorial experiments and meta-analysis of field observations implied that *C. watsonii* will thrive in the projected warmer and P-limited ocean. Moreover, in the context of global climate change, other major environmental stressors like elevated pCO_2 and increasing light intensities have also been found to enhance the growth and CO_2 and N_2 fixation of *C. watsonii* (Garcia et al. 2013*a*; Gradoville et al. 2014). All these findings lead to a reasonable hypothesis that *C. watsonii* will be selected and successful in the future ocean. In addition, under P-limited conditions, elevated temperature resulted in the decrease of the cell size of *C. watsonii*, which may alter its sinking efficiency and hence the C and N export in the future ocean (Fig. 1B). The interactive effects of multiple environmental factors associated with climate change on *C. watsonii*, and the ecological consequences of physiological changes of *C. watsonii* to the community structure, trophic interaction and C and N cycling of the microbial food web in the future warming ocean should be further explored.

References

Bench, S. R., I. Frank, J. Robidart, and J. P. Zehr. 2016. Two subpopulations of *Crocosphaera watsonii* have distinct distributions in the North and South Pacific. Environ. Microbiol. **18**: 514–524. doi:10.1111/1462-2920.13180

Bergman, B., G. Sandh, S. Lin, J. Larsson, and E. J. Carpenter. 2013. *Trichodesmium*—A widespread marine cyanobacterium with unusual nitrogen fixation properties. FEMS Microbiol. Rev. **37**: 286–302. doi:10.1111/j.1574-6976.2012.00352.x

Capone, D. G., J. P. Zehr, H. W. Paerl, B. Bergman, and E. J. Carpenter. 1997. *Trichodesmium*, a globally significant marine cyanobacterium. Science **276**: 1221–1229. doi:10.1126/science.276.5316.1221

Chappell, P. D., and E. A. Webb. 2010. A molecular assessment of the iron stress response in the two phylogenetic clades of *Trichodesmium*. Environ. Microbiol. **12**: 13–27. doi:10.1111/j.1462-2920.2009.02026.x

Chen, M., Y. Lu, N. Jiao, J. Tian, S.-J. Kao, and Y. Zhang. 2019. Biogeographic drivers of diazotrophs in the western Pacific Ocean. Limnol. Oceanogr. **64**: 1403–1421. doi:10.1002/lno.11123

Cheung, S., and others. 2020. Physical forcing controls the basin-scale occurrence of nitrogen-fixing organisms in the North Pacific Ocean. Global Biogeochem. Cycl. **34**: e2019GB006452. doi:10.1029/2019GB006452

Clark, L. L., E. D. Ingall, and R. Benner. 1998. Marine phosphorus is selectively remineralized. Nature **393**: 426–426. doi:10.1038/30881

Collins, M., and others. 2013. Long-term climate change: Projections, commitments and irreversibility, p. 1029–1136. *In* Climate change 2013—The physical science basis: Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge Univ. Press.

Doney, S. C. 2006. Plankton in a warmer world. Nature **444**: 695–696. doi:10.1038/444695a

Dron, A., S. Rabouille, P. Claquin, P. Chang, V. Raimbault, A. Talec, and A. Sciandra. 2012. Light-dark (12:12 h) quantification of carbohydrate fluxes in *Crocosphaera watsonii*. Aquat. Microb. Ecol. **68**: 43–65. doi:10.3354/ame01600

Dyhrman, S. T., and S. T. Haley. 2006. Phosphorus scavenging in the unicellular marine diazotroph *Crocosphaera watsonii*. Appl. Environ. Microbiol. **72**: 1452–1458. doi:10.1128/AEM.72.2.1452-1458.2006

Elser, J. J., D. R. Dobberfuhl, N. A. MacKay, and J. H. Schampel. 1996. Organism size, life history, and N:P stoichiometry: Toward a unified view of cellular and ecosystem processes. Bioscience **46**: 674–684. doi:10.2307/1312897

Finkel, Z. V., J. Beardall, K. J. Flynn, A. Quigg, T. A. V. Rees, and J. A. Raven. 2009. Phytoplankton in a changing world: Cell size and elemental stoichiometry. J. Plankton Res. **32**: 119–137. doi:10.1093/plankt/fbp098

Fu, F. X., E. Yu, N. S. Garcia, J. Gale, Y. Luo, E. A. Webb, and D. A. Hutchins. 2014. Differing responses of marine N_2 fixers to warming and consequences for future diazotroph community structure. Aquat. Microb. Ecol. **72**: 33–46. doi:10.3354/ame01683

Gallon, J. R., and others. 2002. Maximum rates of N_2 fixation and primary production are out of phase in a developing cyanobacterial bloom in the Baltic Sea. Limnol. Oceanogr. **47**: 1514–1521. doi:10.4319/lo.2002.47.5.1514

Garcia, N. S., F. X. Fu, C. L. Breene, E. K. Yu, P. W. Bernhardt, M. R. Mulholland, and D. A. Hutchins. 2013*a*. Combined effects of CO_2 and light on large and small isolates of the unicellular N_2-fixing cyanobacterium *Crocosphaera watsonii* from the western tropical Atlantic Ocean. Eur. J. Phycol. **48**: 128–139. doi:10.1080/09670262.2013.773383

Garcia, N. S., F. X. Fu, and D. A. Hutchins. 2013*b*. Colimitation of the unicellular photosynthetic diazotroph *Crocosphaera watsonii* by phosphorus, light, and carbon dioxide. Limnol. Oceanogr. **58**: 1501–1512. doi:10.4319/lo.2013.58.4.1501

Garcia, N. S., F. X. Fu, P. N. Sedwick, and D. A. Hutchins. 2015. Iron deficiency increases growth and nitrogen-fixation rates of phosphorus-deficient marine cyanobacteria. ISME J. **9**: 238–245. doi:10.1038/ismej.2014.104

Gillooly, J. F., and others. 2005. The metabolic basis of whole-organism RNA and phosphorus content. Proc. Natl. Acad. Sci. U.S.A. **102**: 11923–11927. doi:10.1073/pnas.0504756102

Gómez-Baena, G., O. A. Rangel, A. López-Lozano, J. M. García-Fernández, and J. Diez. 2009. Stress responses in *Prochlorococcus* MIT9313 vs. SS120 involve differential expression of genes encoding proteases ClpP, FtsH and Lon. Res. Microbiol. **160**: 567–575. doi:10.1016/j.resmic.2009.08.009

Gómez-García, M. A. R., M. Losada, and A. Serrano. 2003. Concurrent transcriptional activation of *ppa* and *ppx* genes by phosphate deprivation in the cyanobacterium *Synechocystis* sp. strain PCC 6803. Biochem. Biophys. Res. Commun. **302**: 601–609. doi:10.1016/S0006-291X(03)00162-1

Gradoville, M. R., A. E. White, and R. M. Letelier. 2014. Physiological response of *Crocosphaera watsonii* to enhanced and fluctuating carbon dioxide conditions. PLoS One **9**: e110660. doi:10.1371/journal.pone.0110660

Guo, C., H. Liu, J. Yu, S. Zhang, and C. J. Wu. 2014. Role of microzooplankton grazing in regulating phytoplankton biomass and community structure in response to atmospheric aerosol input. Mar. Ecol. Prog. Ser. **507**: 69–79. doi:10.3354/meps10809

Hama, T., T. Miyazaki, Y. Ogawa, T. Iwakuma, M. Takahashi, A. Otsuki, and S. Ichimura. 1983. Measurement of photosynthetic production of a marine phytoplankton population using a stable ^{13}C isotope. Mar. Biol. **73**: 31–36. doi:10.1007/BF00396282

Hutchins, D. A., F. X. Fu, Y. Zhang, M. E. Warner, Y. Feng, K. Portune, P. W. Bernhardt, and M. R. Mulholland. 2007. CO_2 control of *Trichodesmium* N_2 fixation, photosynthesis, growth rates, and elemental ratios: Implications for past, present, and future ocean biogeochemistry. Limnol. Oceanogr. **52**: 1293–1304. doi:10.4319/lo.2007.52.4.1293

Hutchins, D. A., and P. W. Boyd. 2016. Marine phytoplankton and the changing ocean iron cycle. Nat. Clim. Change **6**: 1072–1079. doi:10.1038/nclimate3147

Hutchins, D. A., and F. X. Fu. 2017. Microorganisms and ocean global change. Nat. Microbiol. **2**: 17058. doi:10.1038/nmicrobiol.2017.58

Jiang, H. B., and others. 2018. Ocean warming alleviates iron limitation of marine nitrogen fixation. Nat. Clim. Change **8**: 709–712. doi:10.1038/s41558-018-0216-8

Karl, D., R. Letelier, L. Tupas, J. Dore, J. Christian, and D. Hebel. 1997. The role of nitrogen fixation in biogeochemical cycling in the subtropical North Pacific Ocean. Nature **388**: 533–538. doi:10.1038/41474

Karl, D., R. Bidigare, and R. Letelier. 2001. Long-term changes in plankton community structure and productivity in the North Pacific Subtropical Gyre: The domain shift hypothesis. Deep Sea Res. II Top. Stud. Oceanogr. **48**: 1449–1470. doi:10.1016/S0967-0645(00)00149-1

Karl, D. M. 2014. Microbially mediated transformations of phosphorus in the sea: New views of an old cycle. Ann. Rev. Mar. Sci. **6**: 279–337. doi:10.1146/annurev-marine-010213-135046

Lin, S., R. W. Litaker, and W. G. Sunda. 2016. Phosphorus physiological ecology and molecular mechanisms in marine phytoplankton. J. Phycol. **52**: 10–36. doi:10.1111/jpy.12365

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2_T^{-\Delta\Delta C}$ method. Methods **25**: 402–408. doi:10.1006/meth.2001.1262

Martínez-Pérez, C., and others. 2016. The small unicellular diazotrophic symbiont, UCYN-A, is a key player in the marine nitrogen cycle. Nat. Microbiol. **1**: 16163. doi:10.1038/nmicrobiol.2016.163

Masuda, T., G. Bernát, M. Bečková, E. Kotabová, E. Lawrenz, M. Lukeš, J. Komenda, and O. Prášil. 2018. Diel regulation

of photosynthetic activity in the oceanic unicellular diazotrophic cyanobacterium *Crocosphaera watsonii* WH8501. Environ. Microbiol. **20**: 546–560. doi:10.1111/1462-2920.13963

Mohr, W., M. P. Intermaggio, and J. LaRoche. 2010. Diel rhythm of nitrogen and carbon metabolism in the unicellular, diazotrophic cyanobacterium *Crocosphaera watsonii* WH8501. Environ. Microbiol. **12**: 412–421. doi:10.1111/j.1462-2920.2009.02078.x

Moisander, P. H., R. A. Beinart, I. Hewson, A. E. White, K. S. Johnson, C. A. Carlson, J. P. Montoya, and J. P. Zehr. 2010. Unicellular cyanobacterial distributions broaden the oceanic N_2 fixation domain. Science **327**: 1512–1514. doi:10.1126/science.1185468

Montoya, J. P., M. Voss, P. Kahler, and D. G. Capone. 1996. A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. Appl. Environ. Microbiol. **62**: 986–993. doi:10.1128/aem.62.3.986-993.1996

Mulholland, M. R. 2007. The fate of nitrogen fixed by diazotrophs in the ocean. Biogeosciences **4**: 37–51. doi:10.5194/bg-4-37-2007

Mulholland, M. R., and P. W. Bernhardt. 2005. The effect of growth rate, phosphorus concentration, and temperature on N_2 fixation, carbon fixation, and nitrogen release in continuous cultures of *Trichodesmium* IMS101. Limnol. Oceanogr. **50**: 839–849. doi:10.4319/lo.2005.50.3.0839

Pereira, N., I. N. Shilova, and J. P. Zehr. 2016. Molecular markers define progressing stages of phosphorus limitation in the nitrogen-fixing cyanobacterium, *Crocosphaera*. J. Phycol. **52**: 274–282. doi:10.1111/jpy.12396

Pereira, N., I. N. Shilova, and J. P. Zehr. 2019. Use of the high-affinity phosphate transporter gene, *pstS*, as an indicator for phosphorus stress in the marine diazotroph *Crocosphaera watsonii* (*Chroococcales*, *Cyanobacteria*). J. Phycol. **55**: 752–761. doi:10.1111/jpy.12863

Pujo-Pay, M., and P. Raimbault. 1994. Improvement of the wet-oxidation procedure for simultaneous determination of particulate organic nitrogen and phosphorus collected on filters. Mar. Ecol. Prog. Ser. **105**: 203–203, 207. doi:10.3354/meps105203

Qu, P., F. X. Fu, J. D. Kling, M. Huh, X. Wang, and D. A. Hutchins. 2019. Distinct responses of the nitrogen-fixing marine cyanobacterium *Trichodesmium* to a thermally variable environment as a function of phosphorus availability. Front. Microbiol. **10**: 1282. doi:10.3389/fmicb.2019.01282

Reistetter, E. N., K. Krumhardt, K. Callnan, K. Roache-Johnson, J. K. Saunders, L. R. Moore, and G. Rocap. 2013. Effects of phosphorus starvation versus limitation on the marine cyanobacterium *Prochlorococcus* MED4 II: Gene expression. Environ. Microbiol. **15**: 2129–2143. doi:10.1111/1462-2920.12079

Shi, T., I. Ilikchyan, S. Rabouille, and J. P. Zehr. 2010. Genome-wide analysis of diel gene expression in the unicellular N_2-fixing cyanobacterium *Crocosphaera watsonii* WH 8501. ISME J. **4**: 621–632. doi:10.1038/ismej.2009.148

Shiozaki, T., and others. 2017. Basin scale variability of active diazotrophs and nitrogen fixation in the North Pacific, from the tropics to the subarctic Bering Sea. Global Biogeochem. Cycles **31**: 996–1009. doi:10.1002/2017GB005681

Sohm, J. A., C. Mahaffey, and D. G. Capone. 2008. Assessment of relative phosphorus limitation of *Trichodesmium* spp. in the North Pacific, North Atlantic, and the north coast of Australia. Limnol. Oceanogr. **53**: 2495–2502. doi:10.4319/lo.2008.53.6.2495

Sohm, J. A., E. A. Webb, and D. G. Capone. 2011. Emerging patterns of marine nitrogen fixation. Nat. Rev. Microbiol. **9**: 499–508. doi:10.1038/nrmicro2594

Sterner, R. W., and J. J. Elser. 2002. Ecological stoichiometry: The biology of elements from molecules to the biosphere. Princeton University Press. doi:10.1093/plankt/25.9.1183

Stocker, T. F., and others. 2013. Climate change 2013: The physical science basis. *In* Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, v. **1535**. Cambridge University Press.

Tang, W., and N. Cassar. 2019. Data-driven modeling of the distribution of diazotrophs in the global ocean. Geophys. Res. Lett. **46**: 12258–12269. doi:10.1029/2019GL084376

Taniuchi, Y., Y. l. L. Chen, H. Y. Chen, M. L. Tsai, and K. Ohki. 2012. Isolation and characterization of the unicellular diazotrophic cyanobacterium Group C TW3 from the tropical western Pacific Ocean. Environ. Microbiol. **14**: 641–654. doi:10.1111/j.1462-2920.2011.02606.x

Toseland, A., and others. 2013. The impact of temperature on marine phytoplankton resource allocation and metabolism. Nat. Clim. Change **3**: 979–984. doi:10.1038/nclimate1989

Tuit, C., J. Waterbury, and G. Ravizza. 2004. Diel variation of molybdenum and iron in marine diazotrophic cyanobacteria. Limnol. Oceanogr. **49**: 978–990. doi:10.4319/lo.2004.49.4.0978

Turk-Kubo, K., K. Achilles, T. Serros, M. Ochiai, J. Montoya, and J. Zehr. 2012. Nitrogenase (*nifH*) gene expression in diazotrophic cyanobacteria in the Tropical North Atlantic in response to nutrient amendments. Front. Microbiol. **3**: 386. doi:10.3389/fmicb.2012.00386

Tuszynski, J. 2012. caTools: Tools: moving window statistics, GIF, Base64, ROC AUC, etc. R package version 1.17.1; [accessed 2014 April 01] Available from http://CRAN.R-project.org/package=caTools.

Van Mooy, B. A. S., and others. 2009. Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. Nature **458**: 69–72. doi:10.1038/nature07659

Van Mooy, B. A. S., G. Rocap, H. F. Fredricks, C. T. Evans, and A. H. Devol. 2006. Sulfolipids dramatically decrease phosphorus demand by picocyanobacteria in oligotrophic

marine environments. Proc. Natl. Acad. Sci. U.S.A. **103**: 8607–8612. doi:10.1073/pnas.0600540103

Wanner, B. 1993. Gene regulation by phosphate in enteric bacteria. J. Cell. Biochem. **51**: 47–54. doi:10.1002/jcb.240510110

Webb, E. A., R. W. Jakuba, J. W. Moffett, and S. T. Dyhrman. 2007. Molecular assessment of phosphorus and iron physiology in *Trichodesmium* populations from the western Central and western South Atlantic. Limnol. Oceanogr. **52**: 2221–2232. doi:10.4319/lo.2007.52.5.2221

Webb, E. A., I. M. Ehrenreich, S. L. Brown, F. W. Valois, and J. B. Waterbury. 2009. Phenotypic and genotypic characterization of multiple strains of the diazotrophic cyanobacterium, *Crocosphaera watsonii*, isolated from the open ocean. Environ. Microbiol. **11**: 338–348. doi:10.1111/j.1462-2920.2008.01771.x

Wood, S., and M. S. Wood. 2015. Package "mgcv." R package version 1: 29.

Yamaguchi, T., M. Sato, N. Gonda, K. Takahashi, and K. Furuya. 2020. Phosphate diester utilization by marine diazotrophs *Trichodesmium erythraeum* and *Crocosphaera watsonii*. Aquat. Microb. Ecol. **85**: 211–218. doi:10.3354/ame01951

Yang, N., and others. 2021. Warming iron-limited oceans enhance nitrogen fixation and drive biogeographic specialization of the globally important cyanobacterium *Crocosphaera*. Front. Mar. Sci. **8**: 118. doi:10.3389/fmars.2021.628363

Yvon-Durocher, G., M. Dossena, M. Trimmer, G. Woodward, and A. P. Allen. 2015. Temperature and the biogeography of algal stoichiometry. Glob. Ecol. Biogeogr. **24**: 562–570. doi:10.1111/geb.12280

Zehr, J. P. 2011. Nitrogen fixation by marine cyanobacteria. Trends Microbiol. **19**: 162–173. doi:10.1016/j.tim.2010.12.004

Zehr, J. P., and D. G. Capone. 2020. Changing perspectives in marine nitrogen fixation. Science **368**: 10.1126/science.aay9514

Zhu, Z., and others. 2020. Interactions between ultraviolet radiation exposure and phosphorus limitation in the marine nitrogen-fixing cyanobacteria *Trichodesmium* and *Crocosphaera*. Limnol. Oceanogr. **65**: 363–376. doi:10.1002/lno.11304

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Conflict of Interest

None declared.

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